Magnesium Inhibits Nickel-induced Genotoxicity and Formation of Reactive Oxygen

Yun Chul Hong,[1] Seung R. Paik,[2] Hun Jae Lee,[1] Kwan Hee Lee,[1] and Sun Mun Jang[1]

[1]Department of Preventive Medicine; and [2]Department of Biochemistry, Inha University Medical College, Inchon, Korea

Nickel compounds are recognized to cause nasal and lung cancers. Magnesium is an effective protector against nickel-induced carcinogenesis *in vivo*, although its mechanisms of protection remain elusive. The effects of magnesium carbonate on the cytotoxicity and genotoxicity induced by nickel subsulfide were examined with respect to the inhibition of cell proliferation, micronuclei formation, DNA–protein cross-link formation, and intranuclear nickel concentration. The generation of reactive oxygen by nickel chloride was also analyzed by observing 8-hydroxy-deoxyguanosine formation from deoxyguanosine in the presence and absence of magnesium chloride. The suppression of up to 64% of the proliferation of BALB/3T3 fibroblasts by nickel subsulfide (1 µg/ml) was reversed by magnesium. The nickel compound increased not only the number of micronuclei but also the amount of DNA–protein cross-links examined with CHO and BALB/3T3 cells, respectively. These genotoxic effects of nickel were again lessened by magnesium carbonate. In addition, the cellular accumulation of nickel increased 80-fold with nickel subsulfide treatment and decreased with magnesium carbonate treatment. Nickel also enhanced 8-hydroxy-deoxyguanosine formation in the presence of H_2O_2 and ascorbic acid, where magnesium played another suppressive role. In fact, inhibition by magnesium was still observed even in the absence of nickel treatment. These results suggest that the protective role of magnesium in nickel-induced cytotoxicity and genotoxicity can be attributed to its ability to reduce either the intracellular nickel concentration or reactive oxygen formation. *Key words*: genotoxicity, 8-hydroxy-deoxyguanosine, magnesium, nickel, reactive oxygen. *Environ Health Perspect* 105:744–748 (1997)

Epidemiologic studies have shown that nickel compounds cause nasal and lung cancers in exposed workers (*1*). Nickel-containing compounds induced tumors in various experimental animals via several different types of exposure (*2–4*). DNA–protein cross-links and chromosomal aberrations were observed in mammalian cells in culture to which nickel compounds were added (*5,6*). In addition, nickel(II) caused oxidative damage to isolated DNA and chromatin in the presence of hydrogen peroxide, possibly due to the formation of reactive oxygen species (*7*).

Magnesium is recognized as an effective protector against nickel-induced carcinogenesis *in vivo* (*8,9*). This protection may be attributed to a simple competition between nickel and magnesium ions for common intracellular counterparts because these divalent cations resemble each other in terms of their physicochemical properties (*10*). Magnesium, therefore, is suspected to suppress genotoxicity and the formation of reactive oxygen induced by nickel, which ultimately leads to carcinogenesis.

In this report, the effects of magnesium carbonate on the cytotoxicity and the genotoxicity resulting from nickel treatment were examined with respect to inhibition of cell proliferation, micronuclei formation, and DNA–protein cross-link formation. The effect of magnesium on intracellular and intranuclear accumulation of nickel was also investigated. In addition, reactive oxygen generation by nickel chloride was observed in the presence and absence of magnesium chloride by detecting 8-hydroxy-deoxyguanosine (8-OH-dG) formation in a deoxyguanosine (dG) hydroxylation system. It has been clearly demonstrated that magnesium not only inhibits nickel-induced cytotoxicity and genotoxicity but also reduces the intracellular accumulation of nickel. The 8-OH-dG formation generated by H_2O_2 and ascorbic acid even in the absence of nickel was inhibited by magnesium. This fact may indicate that magnesium can participate in the generation of reactive oxygen and its biological consequences.

Materials and Methods

Materials. Nickel subsulfide (Ni_3S_2) with particle size <5 µm was obtained from INCO Canada, Ltd. (Toronto, Ontario). Magnesium carbonate with particle size <2 µm and nickel chloride were purchased from Aldrich Chemical Co. (Milwaukee, WI). Magnesium chloride was from Sigma Chemical Co. (St. Louis, MO). Cell lines such as V79, BALB/3T3, and Chinese hamster ovary (CHO) were provided by the American Type Culture Collection (Rockville, MD). Dulbecco's modified Eagle's medium (DMEM), F-12 medium, Ca-Mg free phosphate buffered saline (PBS), fetal bovine serum, calf serum, trypsin, Giemsa, and proteinase K were obtained from Gibco-BRL (Gaithersburg, MD). Hydrogen peroxide and ascorbic acid were from Merck (Darmstadt, Germany). Hoechst 33258 dye was supplied by Polysciences (Warrington, PA). All other reagents were used in the highest purity available from Sigma.

Cell culture. V79 or BALB/3T3 fibroblast cells were grown to confluence in DMEM to which 5% fetal bovine serum or 10% calf serum were added, respectively. The cells were cultured in a humid incubator supplied with 10% CO_2 at 37°C. CHO fibroblast cells were cultured in F12 medium with 10% fetal bovine serum in the presence of 5% CO_2 at 37°C. A mixture of antibiotics composed of penicillin, streptomycin, and amphotericin B was added to the culture medium at a final concentration of 1%.

Inhibition of cell proliferation. The effects of nickel subsulfide and magnesium carbonate on cell proliferation were examined with the BALB/3T3 fibroblast cells. The cell culture was begun by inoculating 5×10^4 cells/ml in a 25-cm^2 culture flask and continued for 24 hr until the log phase of growth. After another 24 hr of incubation in the presence of metals, the cells were collected and counted with a hemocytometer.

Micronuclei formation. The micronuclei formation was assayed in the CHO fibroblast cells with the cytochalasin B-blocked binucleated cell assay. The cells were grown for 8 hr and treated with nickel subsulfide and magnesium carbonate in the presence of 3 µg/ml of cytochalasin B. After 16 hr of additional incubation, the cells were harvested with 0.25% trypsin treatment and centrifuged at 100*g* for 6 min. The resulting pellet was resuspended and incubated with 3 ml of 0.075 M KCl for 5 min at 37°C. Following another centrifugation under the same conditions, the cells resuspended from the pellet were fixed with Carnoy's fixative (methanol: acetic acid at 3:1) and spread on dry slides. The air-dried slides were stained with Giemsa and scored blindly with a microscope under 1,000-fold magnification. For each measurement, 500 binucleated cells were analyzed to observe the presence of micronuclei.

Address correspondence to Y. C. Hong, Department of Preventive Medicine, Inha University Medical College, 253 Yonghyun-Dong, Nam-Gu, Inchon, 402-751, Korea.

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DNA–protein cross-links. The DNA–protein cross-links (DPCs) were detected with the K-SDS assay described by Zhitkovich and Costa (*11*), with minor modifications. In brief, the cells treated with various chemicals were washed twice and collected with 0.25% trypsinization. Following centrifugation at 600*g*, the resulting pellet was resuspended with PBS to give a final concentration of 10^6 cells/ml. The cells were subjected to lysis with 0.5 ml of 2% SDS and 1 mM PMSF in 20 mM Tris-HCl (pH 7.5) in a total volume of 1.5 ml. The mixture was vigorously vortexed for 10 sec and heated at 65°C for 10 min. After further addition of 0.2 M KCl (0.5 ml) prepared in 20 mM Tris-HCl (pH 7.5), the sample was passed five times through a 21-gauge needle. The K-SDS precipitate was formed by cooling of the sample on ice for 5 min and collected by centrifugation at 5,000*g* for 6 min at 4°C. The resulting pellet was washed twice with the following procedure: the pellet was resuspended with 1 ml of 0.2 M KCl in 20 mM Tris-HCl being passed through the needle five times, heated at 65°C for 10 min, cooled on ice, and centrifuged at 5,000*g* for 6 min at 4°C. This thoroughly washed precipitate was incubated with proteinase K (0.2 mg/ml) at 50°C for 3 hr in 1 ml of reaction mixture containing 0.1 M KCl and 10 mM EDTA in 20 mM Tris-HCl (pH 7.5). The released SDS due to the proteolysis was removed by cooling the sample down on ice in the presence of 100 µg bovine serum albumin as a carrier protein to facilitate the precipitation. The amount of DNA in the supernatant was determined with a fluorescent dye of Hoechst 33258 in a DNA fluorometer (Hoefer Scientific Instruments, San Francisco, CA) with excitation and emission wavelengths at 365 and 460 nm, respectively. The total DNA was determined by measuring free DNA obtained in the supernatants during the several washing steps. Effectiveness of the metals in DPC formation was estimated with a ratio between the percentage of DNA cross-linked to proteins in total DNA of the metal-treated cells and the percentage of DNA in control cells.

Intracellular and intranuclear nickel concentrations. V79 fibroblast cells were used because the inhibitory effect of cell proliferation by nickel subsulfide was not apparent compared with that in either BALB/3T3 cells or CHO cells employed in other studies. These cells were incubated for 24 hr with nickel subsulfide at 1 µg/ml in the presence of various amounts of magnesium carbonate. Cells were washed twice with PBS and harvested with 0.25% trypsinization. Intracellular and intranuclear nickel concentrations were measured with an atomic absorption spectrometer (Varian SpectrAA-200, Victoria, Australia) following microwave (Questron Q45, Mercerville, NJ) digestion with nitric acid.

8-OH-dG. Hydroxylation of deoxyguanosine was performed in reaction mixtures containing 0.75 mM dG, 20 mM H_2O_2, and 8 mM ascorbic acid with 50 mM Tris-HCl (pH 7.4) in the presence of various amounts of nickel chloride and magnesium chloride. The mixtures were incubated for various intervals (3, 24, 48 hr) at 37°C. The generation of 8-OH-dG was analyzed with HPLC using a C18 reversed-phase column at 280 nm. The modified nucleoside was separated from dG under an isocratic gradient using 10% methanol in 50 mM KH_2PO_4 at a flow rate of 1 ml/min. The metal effects on 8-OH-dG formation were represented by ratios of peak areas between 8-OH-dG and dG on chromatograms.

Results

Cytotoxicity and genotoxicity. The cytotoxic effects of the particulate forms of nickel and magnesium on cell proliferation were examined with BALB/3T3 fibroblast cells. The cells were incubated with and without nickel subsulfide at 1 µg/ml in the presence of various amounts of magnesium carbonate (0.6, 1.2, 2.4 µg/ml) to give final molar ratios of 0.25, 0.5, and 1.0. Whereas the magnesium itself did not affect cell growth, the nickel subsulfide definitely suppressed cell proliferation to 64% (Fig. 1). This suppression, however, was slowly recovered with the magnesium in a dose-dependent manner.

When micronuclei and DPC formation were observed with CHO and BALB/3T3 fibroblast cells, respectively, the nickel compound exhibited significant increases not only in numbers of micronuclei but also in DPCs over controls (Tables 1,2). These genotoxic effects of nickel were again protected by the magnesium carbonate, although the magnesium itself showed little effect on the assays. The nickel subsulfide at 1 µg/ml increased the number of micronuclei from 12 to 54 in controls out of 500 binucleated cells. This number was reduced to 34 upon magnesium carbonate cotreatment at 2.4 µg/ml. The magnesium itself, however, did not change the numbers. This type of protection by magnesium was also found in the experiment on DPC formation. The DPC coefficient of 1.63 obtained in the presence of nickel was decreased to 1.39 with magnesium subsulfide cotreatment at 2.4 µg/ml.

Measurement of nickel concentrations. Intracellular and intranuclear nickel concentrations in V79 fibroblast cells were determined after 24 hr of incubation with the metals (Fig. 2). Treatment with nickel subsulfide increased the intracellular nickel concentration by 80-fold over that in control cells. This accumulation, however, was gradually decreased to 3.09 ng from 8.61 ng/10^4 cells as the concentrations of magnesium carbonate cotreated with the nickel were raised to 2.4 µg/ml. This decrease was more prominent in the case of intranuclear nickel concentrations in which 0.70 ng of

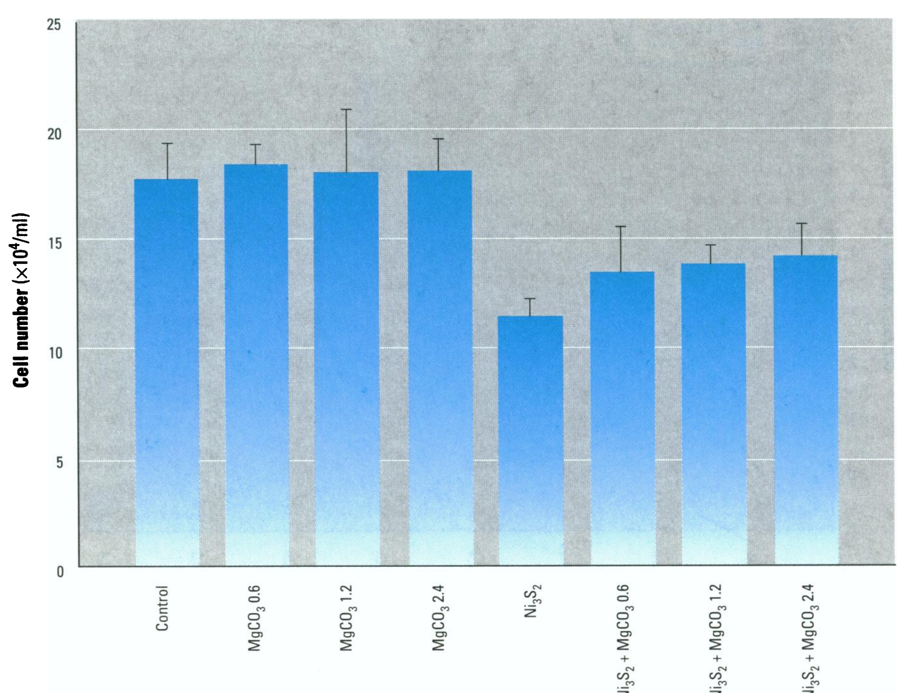

Figure 1. Inhibition of BALB/3T3 cell proliferation after 24 hr incubation in the presence of magnesium carbonate (µg/ml) alone or with nickel subsulfide (1.0 µg/ml). Error bars indicate standard deviation.

Table 1. Micronuclei formation in CHO cells after 16 hr of incubation with the metals in the presence of cytochalasin B

	Number of micronuclei					
Treatment	1	2	3	4	5	Micronuclei/500 binucleated cells
Control	10	1	0	0	0	12
$MgCO_3$ 0.6 µg/ml	7	2	1	0	0	14
$MgCO_3$ 1.2 µg/ml	9	1	0	0	0	11
$MgCO_3$ 2.4 µg/ml	7	2	0	0	0	11
Ni_3S_2 1 µg/ml	24	6	3	1	1	54
Ni_3S_2 1 µg/ml + $MgCO_3$ 0.6 µg/ml	21	7	4	2	0	55
Ni_3S_2 1 µg/ml + $MgCO_3$ 1.2 µg/ml	20	3	1	0	0	29
Ni_3S_2 1 µg/ml + $MgCO_3$ 2.4 µg/ml	19	4	1	1	0	34

Table 2. DNA–protein cross-link formation in BALB/3T3 cell after 24 hr in the presence of the metals

Treatment	Protein-cross-linked DNA %[a]	DPC coefficient[b]
Control	2.05 ± 0.35	1.00
$MgCO_3$ 0.6 µg/ml	2.15 ± 0.92	1.05
$MgCO_3$ 1.2 µg/ml	2.40 ± 0.42	1.17
$MgCO_3$ 2.4 µg/ml	1.65 ± 0.35	0.80
Ni_3S_2 1 µg/ml	3.35 ± 0.35	1.63
Ni_3S_2 1 µg/ml + $MgCO_3$ 0.6 µg/ml	3.30 ± 0.57	1.61
Ni_3S_2 1 µg/ml + $MgCO_3$ 1.2 µg/ml	2.85 ± 0.07	1.39
Ni_3S_2 1 µg/ml + $MgCO_3$ 2.4 µg/ml	2.85 ± 0.07	1.39

DPC, DNA–protein cross-link.
[a]Protein-cross-linked DNA/total DNA; values are obtained in duplicate measurements (mean ± standard deviation).
[b]Protein-cross-linked DNA (%) in metal-treated cells/protein-cross-linked DNA (%) in control cells.

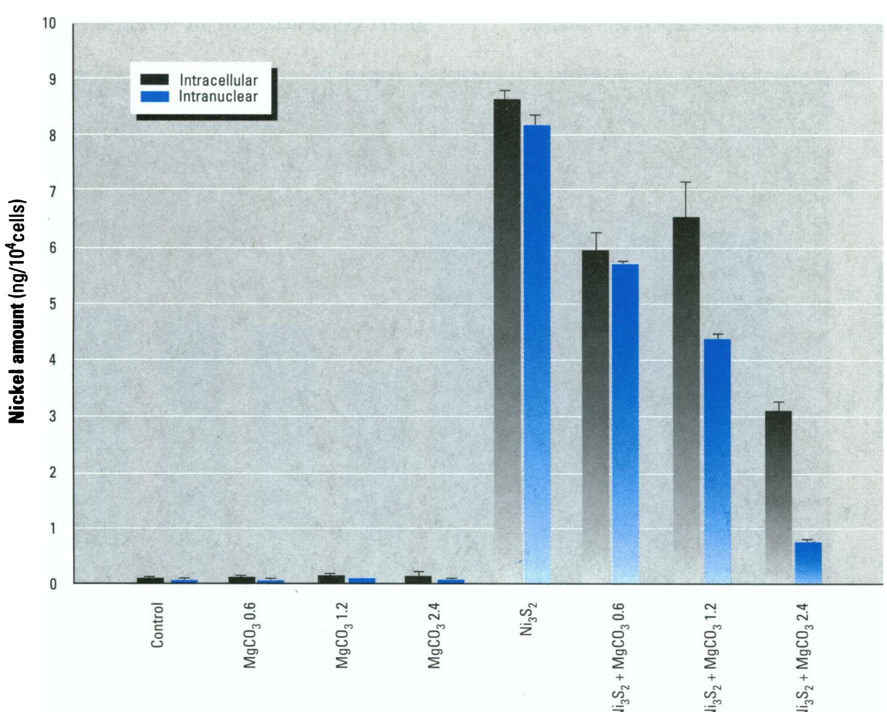

Figure 2. Intracellular and intranuclear nickel concentration of V79 cells after 24 hr in the presence of magnesium carbonate (µg/ml) alone or with nickel subsulfide (1.0 µg/ml). Error bars indicate standard deviation.

nickel /10^4 cells was found at a magnesium carbonate treatment of 2.4 µg/ml.

8-OH-dG formation. In order to pursue a possible protective role of magnesium against nickel-induced reactive oxygen formation, the metal effects were investigated with hydroxylation of deoxyguanosine in the presence of H_2O_2 and ascorbic acid. It was reported that magnesium deficiency caused endothelial cells to be more sensitive to free radical-induced oxidative damage *in vitro* (*12*), which also prompted us to investigate the role of magnesium during 8-OH-dG formation. The hydroxylation was increased to 2.02% with 24 hr of incubation with H_2O_2 and ascorbic acid. The amount of modified nucleoside was decreased to 0.86% during an additional 24 hr of incubation, which might be partly due to deglycosylation (*13*). This modification was enhanced by $NiCl_2$ in a concentration-dependent manner (Table 3). Magnesium chloride clearly inhibited the 8-OH-dG formation produced by nickel at its final concentration of 20 mM in the presence of H_2O_2 and ascorbic acid (Table 4). In addition, this inhibited 8-OH-dG formation was still obvious even in the absence of the nickel (Table 5). At 40 mM $MgCl_2$, the hydroxylated product was found to be 0.50%, markedly decreased from 2.02% in controls. This fact clearly suggests that magnesium is involved in the process of formation and/or the degeneration of reactive oxygen species independent of the nickel compound. In other words, magnesium played an antioxidative function in this experiment.

Discussion

This study was carried out to examine the genotoxic effects of nickel in cultured cells, to evaluate the protective effects of magnesium against nickel toxicity, and finally to find the effects of metals on reactive oxygen formation. Much evidence supports a close relationship between nickel toxicity and carcinogenicity, which involves the formation of reactive oxygen species inside cells (*7,14,15*). We have clearly demonstrated in this study that the genotoxicity of Ni_3S_2 assessed by micronuclei formation and DNA–protein cross-link formation was markedly reduced by the magnesium

Table 3. 8-Hydroxy-deoxyguanosine/total deoxyguanosine change by nickel chloride in the presence of H_2O_2 and ascorbic acid

Treatment	3 hr	24 hr	48 hr
Control	1.21%	2.02%	0.86%
$NiCl_2$ 5 mM	1.44%	2.27%	1.33%
$NiCl_2$ 10 mM	1.75%	2.96%	1.58%
$NiCl_2$ 20 mM	2.28%	3.23%	1.56%

carbonate. This effect differs from the well-known inhibition of soluble Mg^{2+} salts on nickel-induced genotoxicity because these studies were carried out with 0.6–2.4 µg/ml particulate $MgCO_3$ in cell culture media that already contained the magnesium ion at concentrations of 14.6–19.7 µg/ml. This protection is suspected to be partly due to reduced intracellular nickel concentration because it has already been reported that the nuclear nickel concentrations and the nickel-induced DNA damage were mutually dependent (*16*). In addition, we showed that 8-OH-dG formation by soluble $NiCl_2$ in a dG hydroxylation system as an indicator of the formation of reactive oxygen species was also suppressed by magnesium chloride.

Since these two divalent cations—Ni^{2+} and Mg^{2+}—share many physicochemical properties (*10*), the magnesium effects on intracellular and intranuclear nickel concentrations were somewhat expected. It is certainly possible that there is simple competition between the cations for either the transport process or various intracellular target molecules after being imported through phagocytosis of the metals and solubilized. It is widely accepted that cells phagocytizing particulate compounds obtain a high concentration of biologically available ions inside as a result of dissolving the particle present in intracellular vacuoles (*17*). The uptake and solubilization of the nickel compounds are very important with respect to the mechanism of nickel carcinogenesis (*18–20*). The inhibitory effects of magnesium on the various nickel-induced biological phenomenon caused by nickel in this study could be due to an interference of undissolved $MgCO_3$ with the phagocytic uptake and the solubilization of Ni_3S_2 because these two metals are slightly soluble in the cell culture medium.

The Ni_3S_2, under conditions that inhibit cell growth, also induced a significant amount of micronuclei and DNA–protein cross-link formation. The micronuclei formation analyzed with the cytokinesis-block method is known to be related to the phenomenon of chromosomal aberration caused by radiation and chemical compounds (*21–23*). In fact, various nickel compounds were responsible for chromosomal aberrations in both *in vivo* and *in vitro* systems (*24,25*). DNA–protein cross-links frequently produced by many carcinogens are considered to be critical lesions leading to genotoxicity because they impede the activities of proteins involved in DNA replication, transcription, and repair (*11,26,27*). Since these complexes persist even after the removal of the cross-linking agents and are difficult to be repaired, they would cause permanent DNA damage (*28–31*). It has been shown that the DNA–protein cross-link produced by nickel resulted from oxygen radicals (*32–34*). It is unlikely for nickel by itself to cross-link DNA and protein directly (*35*). We obtained SDS-resistant DNA–protein cross-links, which indicate that these cross-links were formed by oxidative stress instead of the metal itself. The fact that the inhibition of DNA repair by Ni^{2+} is reversed by Mg^{2+} provides further evidence for competition between these metal ions (*4*).

Recent studies on nickel carcinogenesis indicate that reactive oxygen species were implicated in not only nickel-induced lipid peroxidation but also in DNA damage caused by 8-OH-dG formation, which interferes with DNA replication (*13,36,37*). The 8-OH-dG formed by hydroxylation at the C8 position of the guanosine residues in DNA has attracted special interest with respect to carcinogenesis involving reactive oxygen species (*38*).

By following the method of Kasai and Nishimura (*38*), we have tried to find whether soluble $NiCl_2$ in the presence of H_2O_2 and ascorbic acid can enhance the oxidation of dG and whether soluble magnesium can affect the oxidation. Our results show that nickel facilitates 8-OH-dG production, the major oxidative product from dG with H_2O_2 and ascorbic acid, in time- and concentration-dependent manners, which indicates that the metal is directly responsible for the formation of reactive oxygen species. $MgCl_2$, on the other hand, decreases the enhancement of 8-OH-dG formation by nickel. We have also observed the capability of magnesium to reduce dG hydroxylation, even in the absence of nickel treatment. The action of the magnesium ion seems to occur directly at the level of reactive oxygen formation without any involvement of nickel ions. Although the exact molecular mechanisms underlying these reactions remain to be investigated, this study shows that magnesium deficiency can enhance free radical-induced oxidation *in vivo* (*12,39*). Our results, therefore, suggest that magnesium is an antioxidant.

In conclusion, we show strong and consistent relationships among cell growth inhibition, micronuclei formation, and DNA–protein cross-link formation in terms of the cytotoxicity and the genotoxicity induced by nickel. In addition, these nickel toxicities are suppressed by particulate $MgCO_3$. Reactive oxygen formation induced by $NiCl_2$ is also reduced by $MgCl_2$ in the dG hydroxylation system. These protective roles of magnesium, therefore, may be explained by its ability to reduce not only intracellular nickel concentrations but also reactive oxygen formation.

Table 4. 8-Hydroxy-deoxyguanosine/total deoxyguanosine change by nickel chloride and magnesium chloride in the presence of H_2O_2 and ascorbic acid

Treatment	3 hr	24 hr	48 hr
Control	1.21%	2.02%	0.86%
$NiCl_2$ 20 mM	2.28%	3.23%	1.56%
$NiCl_2$ 20 mM + $MgCl_2$ 10 mM	1.62%	2.21%	1.17%
$NiCl_2$ 20 mM + $MgCl_2$ 20 mM	1.52%	1.98%	1.00%
$NiCl_2$ 20 mM + $MgCl_2$ 40 mM	1.06%	1.81%	0.82%

Table 5. 8-Hydroxy-deoxyguanosine/total deoxyguanosine change by magnesium chloride in the presence of H_2O_2 and ascorbic acid

Treatment	24 hr
Control	2.02%
$MgCl_2$ 10 mM	1.67%
$MgCl_2$ 20 mM	1.35%
$MgCl_2$ 40 mM	0.50%

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